

## *Inhibitors of Apoptosis*

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### CROSS-REFERENCE TO RELATED APPLICATION

*10A1* ~~This application is a continuation under 35 USC 120 of USSN 08/512,946, filed 08/08/1995.~~

### INTRODUCTION

#### Field of the Invention

The field of this invention is human proteins involved in the inhibition of apoptosis, or programmed cell death.

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#### Background

Cellular apoptosis, or programmed cell death, may be initiated by a variety of different stimuli including viral infection, certain cell-culture conditions, cell-cell signaling, cytokines, etc. Elucidation of signal transduction pathways leading to apoptosis would provide valuable insight into a variety of pathogenic mechanisms. Accordingly, the ability to exogenously modulate the induction of apoptosis would yield therapeutic application for numerous clinical indications. In addition, components of such pathways would provide valuable targets for automated, cost-effective, high throughput drug screening and hence would have immediate application in domestic and international pharmaceutical and biotechnology drug development programs.

#### Relevant Literature

Rothe *et al.* (1994) Cell 78, 681-692, report the existence of tumor necrosis factor (TNF) receptor associated proteins which co-immunoprecipitate with a TNF receptor; see also Rothe, *et al.*, pending US patent application Serial No: 08/446,915. Roy, *et al.* (1995) Cell 80, 167-178 disclose the gene for a human neuronal apoptosis

inhibitory protein. Birnbaum et al. (1994) J Virol 68, 2521-2528 disclose an inhibitor of apoptosis (iap) gene, Op-iap from the *Orgyia pseudotsugata* nuclear polyhedrosis virus (OpMNPV) with sequence similarity to two other viral genes: Cp-iap derived from *Cydia pomonella* granulosis virus (CpGV), and iap derived from the *Autographa californica* nuclear polyhedrosis virus (AcMNPV). Clem and Miller (1994), in Apoptosis II: The Molecular Basis of Apoptosis in Disease, pp 89-110, Cold Spring Harbor Laboratory Press, provide a recent review of apoptosis regulation by insect viruses.

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#### SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to novel human cellular inhibitor of apoptosis proteins (c-IAP). The subject proteins comprise a series of defined structural domain repeats and/or a RING finger domain; in particular, at least two of a first domain repeat comprising SEQUENCE ID NO: 5 or 6; a second domain repeat comprising SEQUENCE ID NO: 7 or 8; and a third domain repeat comprising SEQUENCE ID NO: 9 or 10; and/or a RING finger domain comprising SEQUENCE ID NO: 11 or 12, or a consensus sequences derived from these human genes. The proteins provide a c-IAP specific function, with preferred proteins being capable of modulating the induction of apoptosis; for example, by binding a human tumor necrosis factor receptor associated factor, TRAF. The compositions include nucleic acids which encode the subject c-IAP and hybridization probes and primers capable of hybridizing with the disclosed c-IAP genes.

The invention includes methods of using the subject compositions in therapy (e.g. gene therapy to enhance expression of a c-IAP gene), in diagnosis (e.g. genetic hybridization screens for c-IAP gene mutations, and in the biopharmaceutical industry (e.g. reagents for increasing yields of recombinant protein by enhancing host cell survival in culture, for screening chemical libraries for lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated with apoptosis regulation, etc.).

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#### DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods and compositions relating to novel cellular inhibitor of apoptosis proteins (c-IAPs). The nucleotide sequence of a natural cDNA

encoding human c-IAP is shown as SEQUENCE ID NO:1 and the full conceptual translate is shown as SEQUENCE ID NO:2. The nucleotide sequence of another natural cDNA encoding human c-IAP2 is shown as SEQUENCE ID NO:3 and the full conceptual translate is shown as SEQUENCE ID NO:4. The human c-IAPs of the invention include incomplete translates of SEQUENCE ID NOS:1 and 3 or deletion mutants of SEQUENCE ID NOS: 2 and/or 4, which translates or deletions mutants have at least one of the human c-IAP specific activities described herein. In addition, the invention provides nonhuman mammalian homologs of the disclosed human c-IAPs. These homologs are encoded by natural cDNAs which are capable of specifically hybridizing with one or more of the disclosed human cDNAs under hybridization conditions describe below and are isolated using the methods and reagents described herein. For example, the amino acid sequence of a murine homolog of c-IAP1, and the sequence its cDNA are shown in SEQUENCE ID NOS: 14 and 13.

15 The subject proteins comprise a series of defined structural domain repeats and/or a RING finger domain shown to be necessary for human c-IAP specific function; generally including at least two of: a first domain repeat comprising SEQUENCE ID NO: 5, 6 or a consensus of 5 and 6, a second domain repeat comprising SEQUENCE ID NO: 7, 8 or a consensus of 7 and 8, and a third domain 20 repeat comprising SEQUENCE ID NO: 9, 10 or a consensus of 9 and 10; and/or a RING finger domain comprising SEQUENCE ID NO: 11, 12 or a consensus of 11 and 12. Preferred domain repeat containing c-IAPs contain each of the three domain repeats. More preferred c-IAPs comprise the three domain repeats and the C-terminal RING finger. To secure or optimize the requisite function for the protein, the repeats 25 are usually preceded (N-terminally) and separated by intervening regions of about 10 to about 100 residues, which regions preferably derive from those found in the natural c-IAP1 and c-IAP2 translates. Similarly, the RING finger domain of RING finger domain containing c-IAPs containing proteins is usually preceded by an N-terminal region of about 10 to 300 residues, usually 100 to 300 residues, which region 30 preferably derives from those found in the natural c-IAP1 and c-IAP2 translates.

The proteins provide a human c-IAP1 or c-IAP2 (c-IAP1/2) specific activity or function which may be determined by convenient in vitro, cell-based, or in vivo assays. Preferred proteins are capable of modulating the induction of apoptosis. Such

activity or function may be demonstrated in cell culture (e.g. cell transfections) or in animals (e.g. *in vivo* gene therapy, transgenics). c-IAP1/2 specific function can also be demonstrated by specific binding to a c-IAP1/2 specific binding target, including natural binding targets and nonnatural targets such as c-IAP1/2 specific antibodies.

5 For example, c-IAPs comprising at least two of SEQUENCE ID NOS: 6, 7 and 8 are capable of specifically binding human tumor necrosis factor receptor associated factors 1 and 2 (TRAF1 and TRAF2) in simple in vitro binding assays. Finally, specific function can be assayed immunologically by the ability of the subject protein to elicit a c-IAP1/2 specific antibody in a rodent or rabbit. Generally, human

10 c-IAP1/2-specificity of the binding agent is shown by binding equilibrium constants (usually at least about  $10^7 \text{ M}^{-1}$ , preferably at least about  $10^8 \text{ M}^{-1}$ , more preferably at least about  $10^9 \text{ M}^{-1}$ ). A wide variety of cell-based and cell-free assays may be used to demonstrate human c-IAP1/2-specific binding; preferred are rapid in vitro, cell-free assays such as mediating or inhibiting human c-IAP1/2-protein (e.g. human c-IAP1-

15 TRAF2) binding, immunoassays, etc.

The claimed human c-IAP proteins are isolated, partially pure or pure and are typically recombinantly produced. An "isolated" protein for example, is

unaccompanied by at least some of the material with which it is associated in its natural state and constitutes at least about 2%, and preferably at least about 5% by

20 weight of the total protein in a given sample; a partially pure protein constitutes at least about 10%, preferably at least about 30%, and more preferably at least about 60% by weight of the total protein in a given sample; and a pure protein constitutes at least about 70%, preferably at least about 90%, and more preferably at least about 95% by weight of the total protein in a given sample. A wide variety of molecular  
25 and biochemical methods are available for generating and expressing the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, et al. Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art..

30 The invention provides human c-IAP1/2-specific binding agents including substrates, natural intracellular binding targets, etc. and methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, human c-IAP1/2-specific agents are useful in a variety of

diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving human c-IAP1/2, e.g. apoptosis. Novel human c-IAP1/2-specific binding agents include human c-IAP1/2-specific antibodies and other natural intracellular binding agents identified with assays 5 such as one- and two-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries, etc.

The invention also provides nucleic acids encoding the subject proteins, which nucleic acids may be part of human c-IAP1/2-expression vectors and may be incorporated into recombinant cells for expression and screening, transgenic animals 10 for functional studies (e.g. the efficacy of candidate drugs for disease associated with c-IAP1/2 mediated signal transduction), etc., and nucleic acid hybridization probes and replication/amplification primers having a human c-IAP1/2 cDNA specific sequence contained in SEQUENCE ID NO:1 or 3. Nucleic acids encoding human c-IAP1/2 are isolated from eukaryotic cells, preferably human cells, by screening 15 cDNA libraries with probes or PCR primers derived from the disclosed human c-IAP1/2 cDNA.

In addition, the invention provides nucleic acids sharing sufficient sequence similarity with that of the disclosed human c-IAP1/2 cDNAs to effect hybridization thereto. Such human c-IAP1/2 cDNA homologs are capable of hybridizing to the 20 human c-IAP1/2-encoding nucleic acid defined by SEQUENCE ID NO: 1 or 3 under stringency conditions characterized by a hybridization buffer comprising 30% formamide in 5 x SSPE (0.18 M NaCl, 0.01 M NaPO<sub>4</sub>, pH7.7, 0.001 M EDTA) buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with the 0.2 x SSPE. Preferred nucleic acids will hybridize in a hybridization buffer 25 comprising 50% formamide in 5 x SSPE buffer at a temperature of 42°C and remain bound when subject to washing at 42°C with 0.2 x SSPE buffer at 42°C. Human c-IAP1/2 cDNA homologs can also be characterized by BLASTX (Altschul et al. 1990) Basic Local Alignment Search Tool, J Mol Biol 215, 403-410) probability scores. Using this nucleic acid sequence search program BLASTX, complete coding 30 region human c-IAP1/2 cDNA homologs provide a Probability P(N) score of less than 1.0e-200. More preferred nucleic acids encode c-IAPs with at least about 50%, preferably at least about 60%, more preferably at least 70% pair-wise identity to at least one of SEQUENCE ID NOS: 2 and 4.

The subject nucleic acids are isolated, i.e. constitute at least about 0.5%, preferably at least about 5% by weight of total nucleic acid present in a given fraction. The subject nucleic acids find a wide variety of applications including use as translatable transcripts, hybridization probes, PCR primers, therapeutic nucleic acids, etc.; use in detecting the presence of human c-IAP1/2 genes and gene transcripts, in detecting or amplifying nucleic acids encoding additional human c-IAP1/2 homologs and structural analogs, and in gene therapy applications. When used as expression constructs, the nucleic acids are usually recombinant, meaning they comprise a sequence joined to a nucleotide other than that which it is joined to on a natural chromosome. The subject nucleic acids may be contained within vectors, cells or organisms.

In diagnosis, c-IAP1/2 hybridization probes find use in identifying wild-type and mutant c-IAP1/2 alleles in clinical and laboratory samples. Mutant alleles are used to generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. In therapy, therapeutic c-IAP1/2 nucleic acids are used to modulate cellular expression or intracellular concentration or availability of active c-IAP1/2. A wide variety of indications may be treated, either prophylactically or therapeutically with the subject compositions. For example, where cell-specific apoptosis or other limitation of cell growth is desired, e.g. neoproliferative disease, a reduction in c-IAP1/2 expression is effected by introducing into the targeted cell type c-IAP1/2 nucleic acids which reduce the functional expression of c-IAP1/2 gene products (e.g. nucleic acids capable of inhibiting translation of a c-IAP1/2 protein). Conditions for treatment include restenosis, where vascular smooth muscle cells are involved, inflammatory disease states, where endothelial cells, inflammatory cells and glomerular cells are involved, myocardial infarction, where heart muscle cells are involved, glomerular nephritis, where kidney cells are involved, transplant rejection where endothelial cells are involved, infectious diseases such as HIV infection where certain immune cells and other infected cells are involved, or the like.

These c-IAP1/2 inhibitory nucleic acids are typically antisense: single-stranded sequences comprising complements of the disclosed c-IAP1/2 encoding nucleic acid. Antisense modulation of the expression of a given c-IAP1/2 protein may employ c-IAP1/2 antisense nucleic acids operably linked to gene regulatory sequences. Cell are transfected with a vector comprising a c-IAP1/2 sequence with a promoter

sequence oriented such that transcription of the gene yields an antisense transcript capable of binding to endogenous c-IAP1/2 protein encoding mRNA. Transcription of the antisense nucleic acid may be constitutive or inducible and the vector may provide for stable extrachromosomal maintenance or integration. Alternatively,

5 single-stranded antisense nucleic acids that bind to genomic DNA or mRNA encoding a given c-IAP1/2 protein may be administered to the target cell, in or temporarily isolated from a host, at a concentration that results in a substantial reduction in expression of the targeted protein.

In other indications, e.g. certain hypersensitivities, atrophic diseases, etc., a

10 reduction in apoptosis is desired. In these applications, an enhancement in c-IAP1/2 expression is effected by introducing into the targeted cell type c-IAP1/2 nucleic acids which increase the functional expression of c-IAP1/2 gene products. Conditions for treatment include multiple sclerosis, where certain neuronal cells are involved, inflammatory disease states such as rheumatoid arthritis, where bystander cells are involved, transplant rejection where graft cells are involved, infectious diseases such as HIV infection where certain uninfected host cells are involved, or the like. Such nucleic acids may be c-IAP1/2 expression vectors, vectors which upregulate the functional expression of an endogenous c-IAP1/2 allele, or replacement vectors for targeted correction of c-IAP1/2 mutant alleles.

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and the *Journal of the American Revolution* are the best sources of information on the subject.

20 Various techniques may be employed for introducing of the nucleic acids into  
viable cells. The techniques vary depending upon whether one is using the subject  
compositions in culture or *in vivo* in a host. Various techniques which have been  
found efficient include transfection with a retrovirus, viral coat protein-liposome  
mediated transfection, see Dzau et al., *Trends in Biotech* 11, 205-210 (1993). In some  
25 situations it is desirable to provide the nucleic acid source with an agent which targets  
the target cells, such as an antibody specific for a surface membrane protein on the  
target cell, a ligand for a receptor on the target cell, etc. Where liposomes are  
employed, proteins which bind to a surface membrane protein associated with  
endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins  
30 or fragments thereof tropic for a particular cell type, antibodies for proteins which  
undergo internalization in cycling, proteins that target intracellular localization and  
enhance intracellular half-life. In liposomes, the nucleic acid concentration in the  
lumen will generally be in the range of about 0.01  $\mu$ M to 10  $\mu$ M. For other

techniques, the concentration and application rate is determined empirically, using conventional techniques to determine desired ranges.

Application of the subject therapeutics may be systemic or local, i.e. administered at the site of interest. Various techniques can be used for providing the 5 subject compositions at the site of interest, such as injection, use of catheters, trocars, projectiles, pluronic gel, stents, sustained drug release polymers or other device which provides for internal access. Where an organ or tissue is accessible because of removal from the patient, such organ or tissue may be bathed in a medium containing the subject compositions, the subject compositions may be painted onto the organ, or 10 may be applied in any convenient way. Systemic administration of the nucleic acid may be effected using naked DNA, lipofection, liposomes with tissue targeting (e.g. antibody).

The invention provides methods and compositions for enhancing the yield of many recombinantly produced proteins, such as tissue plasminogen activator (t-PA), 15 by increasing maximum cell densities and survival time of host production cells in culture. Specifically, cultured cells are transfected with nucleic acids which effect the up-regulation of endogenous c-IAP or the expression of an exogenous c-IAP. For example, nucleic acids encoding functional c-IAP operably linked to a transcriptional promoter are used to over-express the exogenous c-IAP in the host cell (see, 20 experimental section, below). Such transformed cells demonstrate enhanced survival ability at elevated cell densities and over extended culture periods.

The invention provides efficient methods of identifying pharmacological agents or lead compounds for agents active at the level of a human c-IAP1/2 modulatable cellular function, particularly human c-IAP1/2 mediated signal 25 transduction, especially in apoptosis. Generally, these screening methods involve assaying for compounds which modulate a human c-IAP1/2 interaction with a natural c-IAP1/2 binding target. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the 30 reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development. Target indications may include infection, genetic disease, cell growth and regulatory dysfunction, such as neoplasia, inflammation, hypersensitivity, etc.

A wide variety of assays for binding agents are provided including protein-protein binding assays, immunoassays, cell based assays, etc. The human c-IAP1/2 compositions used the methods are usually added in an isolated, partially pure or pure form and are typically recombinantly produced. The human c-IAP1/2 may be part of 5 a fusion product with another peptide or polypeptide, e.g. a polypeptide that is capable of providing or enhancing protein-protein binding, stability under assay conditions (e.g. a tag for detection or anchoring), etc. The assay mixtures comprise a natural intracellular human c-IAP1/2 binding target such as a TRAF. While native binding targets may be used, it is frequently preferred to use portions (e.g. peptides, nucleic 10 acid fragments) thereof so long as the portion provides binding affinity and avidity to the subject human c-IAP1/2 conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including 15 libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, 20 antimicrobial agents, etc. may be used.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the human c-IAP1/2 specifically binds the cellular binding target, portion or analog with a reference binding affinity. The mixture components can be added in any order that provides for the requisite 25 bindings. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4 and 40°C, more commonly between 15° and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening, and are typically between .1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours.

30 After incubation, the agent-influenced binding between the human c-IAP1/2 and one or more binding targets is detected by any convenient way. For cell-free binding type assays, a separation step is often used to separate bound from unbound components. Separation may be effected by precipitation (e.g. TCA precipitation,

immunoprecipitation, etc.), immobilization (e.g on a solid substrate), etc., followed by washing by, for examples, membrane filtration (e.g. Whatman's P-81 ion exchange paper, Polyfiltronic's hydrophobic GFC membrane, etc.), gel chromatography (e.g. gel filtration, affinity, etc.). In addition, one of the components usually comprises or 5 is coupled to a label. A wide variety of labels may be employed - essentially any label that provides for detection of bound protein. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components.

10 For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected. Labels may be directly detected through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc. For example, in the case of radioactive labels, 15 emissions may be detected directly, e.g. with particle counters or indirectly, e.g. with scintillation cocktails and counters.

The following experiments and examples are offered by way of illustration and not by way of limitation.

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## EXPERIMENTAL

The murine cellular inhibitor of apoptosis protein 1 (c-IAP1) was biochemically purified as a TNF-R2 associated protein using coimmunoprecipitation Rothe et al. (1994) *supra*. A large scale protein purification protocol provided material sufficient for peptide sequencing. Fully degenerate oligonucleotides 25 corresponding to two of the isolated peptides were used to specifically amplify a 0.75 kb DNA fragment from mouse CT6 RNA by Reverse Transcription-PCR. This DNA fragment was used to isolate full-length cDNA clones from a mouse CT6 cDNA library by hybridization (50% formamide, 5xSSPE, 42°C; filters washed at 42°C with 0.2XSSPE, where 1xSSPE is 0.18 M NaCl, 0.01 M NaPO<sub>4</sub>, pH7.7, 0.001 M EDTA).

30 DNA sequence analysis predicted an open reading frame encoding a 612 amino acid protein that shows significant sequence similarity (36 % amino acid identity) with the 'inhibitor of apoptosis protein' (IAP) from insect viruses (Clem, R. J. and Miller, L. K., 1994, *supra*) and the human 'neuronal apoptosis inhibitory protein'

(NAIP) (23 % amino acid identity), that is involved in spinal muscular atrophy (SMA) an inherited disease in humans (Roy et ., 1995, *supra*). To obtain the human c-IAP1 gene, the originally amplified mouse DNA fragment was used as a probe to screen a HeLa cDNA library (30% formamide, 5xSSPE, 42°C; filters washed at 42°C with 5 0.2xSSPE). Sequence analysis of the isolated cDNA clones revealed that they correspond to two distinct genes, designated c-IAP1 and c-IAP2. The human c-IAP1 cDNA encodes a protein of 618 amino acids that is 84% identical to murine c-IAP1. The human c-IAP2 cDNA encodes a protein of 604 amino acids that shares a high degree of amino acid identity with both murine and human c-IAP1 (72% and 73%, 10 respectively) and represents another member of the IAP superfamily.

Comparison of the amino acid sequence of members of the IAP superfamily reveals that they are comprised of at least three distinct domains. The N-terminal region of all IAP family members is comprised of 'baculovirus IAP repeat' (BIR) motifs (Birnbaum et al., 1994, *supra*). While the viral proteins contain two repeats, the 15 mammalian homologs (c-IAP1, -2) possess three BIR motifs. Similarly, NAIP contains three BIR repeats. In addition to BIR motifs viral IAPs contain a C-terminal RING finger motif. This Zn-binding domain is also present in c-IAP1 and -2 but not in NAIP. Thus c-IAP1 and -2 define a distinct subfamily within the IAP superfamily that contain three BIR motifs and a RING finger motif. A RING finger domain is also 20 present at the N-terminus of TRAF2 and has been shown to be involved in TRAF2 signal transduction. The RING finger motifs of c-IAP1 and -2 share significant sequence homology with the RING finger domains of viral IAPs but no homology with the TRAF2 RING finger domain besides the conserved cysteine and histidine residues. The region between the BIR domain and the RING finger domain of c-IAP1 25 and -2 is strongly conserved but does not reveal any significant homology to other members of the IAP family or any other proteins in the NCBI database.

A yeast two-hybrid system was used to determine how c-IAP1 and -2 interact with TNF-R2 and/or TRAFs. The following results were obtained indistinguishably for c-IAP1 and c-IAP2. Two-hybrid analysis revealed that c-IAP1 does not directly 30 interact with TNF-R2. However, a direct interaction could be detected between c-IAP1 and TRAF2. The conserved TRAF domain of TRAF2 (amino acids 264-501) is sufficient to mediate this interaction. Consistently, c-IAP1 also interacted with TRAF1. Further analysis demonstrated that the coiled-coil region within the TRAF

domain of TRAF2 (amino acids 251-358) is required for interaction with c-IAP1. In contrast, the C-terminal region of the TRAF domain (amino acids 359-501) that mediates the association of TNF-R2 with TRAF2 is dispensable for interaction of c-IAP1 with TRAF2. Thus c-IAP1 and TNF-R2 bind to non-overlapping docking sites within the TRAF domain of TRAF2. Consistently, c-IAP1 does not interact with TRAF3 (e.g. Cheng et al. (1995), *supra*), which does not contain a coiled-coil region with sequence similarity to TRAF2/TRAF1. Deletion mutagenesis of c-IAP1 indicated that the N-terminal half of the protein containing the three BIR motifs (amino acids 1-336 of c-IAP1 and 1-396 of c-IAP2) is sufficient for interaction with TRAF2 and TRAF1. Similarly, combinations of two of the three BIR motifs e.g. amino acid residues 46-99 and 204-249 of c-IAP1 and 29-82 and 189-234 of c-IAP2, separated by IAP1 derived intervening sequences of varying lengths are assayed for TRAF1 and TRAF2 binding. This indicates that BIR motifs represent a novel protein:protein interaction domain. The RING finger domain of c-IAP1/2 (amino acids 571-618 of c-IAP1 and 557-604 of c-IAP2) is not required for interaction with TRAFs, but rather mediates subsequent steps in the c-IAP1/2 signaling cascade. Similarly, a variety of c-IAP1 derived N-terminal leader sequences fused to the c-IAP1 RING finger domain are used to assay signal transduction mediation. In an analogous situation, the RING finger domain of TRAF2 has been demonstrated to be required for TRAF2-mediated activation of NF- $\kappa$ B.

A transfection based co-immunoprecipitation assay was used to investigate how c-IAP1 interacts with the complex of TNF-R2 and TRAFs. In this system c-IAP1 was N-terminally tagged with a FLAG epitope peptide and expressed in human embryonic 293 cells under the control of a constitutive CMV promotor (pRK vector). The c-IAP1 expression vector was transiently co-transfected into 293 cells with expression vectors for TNF-R2 and TRAFs. After 24-36 h, the cells were harvested and extracts immunoprecipitated with anti-TNF-R2 antibodies, followed by Western analysis with anti-FLAG antibodies. This assay demonstrated that while c-IAP1 associates directly with TRAF1 and TRAF2, its interaction with TNF-R2 is indirect and requires the heterocomplex of TRAF1 and TRAF2. Thus, c-IAP1 is a component of the TNF-R2 (CD40)/TRAF signaling complex.

To determine the functional properties of c-IAP1 transient transfection assays were performed in human rhabdomyosarcoma KYM1 cells. The results indicate that

overexpression of c-IAP1 but not of control vector, TRAF1 or TRAF2 protects KYM1 cells from TNF-induced programmed cell death (apoptosis). Hence, c-IAP1 regulates the cellular response to TNF by modulating TNF responsiveness, e.g. the initiation of an apoptotic or protective program. The transient transfection assay also 5 finds use as a drug screening assay. In this application, candidate agents are screened as above for their ability to modulate the ability of c-IAP1 to downregulate apoptosis.

## EXAMPLES

1. Protocol for human c-IAP1 - TRAF2 binding assay.
- 10 A. Reagents:
  - Neutralite Avidin: 20 µg/ml in PBS.
  - Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
  - Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM  $\beta$ -mercaptoethanol, 1 mg/ml BSA, cocktail of protease 15 inhibitors.
  - $^{32}$ P human c-IAP1 10x stock:  $10^{-8}$  -  $10^{-6}$  M unlabeled human c-IAP1 supplemented with 200,000-250,000 cpm of labeled human c-IAP1/21 (Beckman counter). Place in the 4°C microfridge during screening.
  - 20 - Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2 mM NaVo<sub>3</sub> (Sigma # S-6508) in 10 ml of PBS.
  - TRAF2:  $10^{-8}$  -  $10^{-5}$  M biotinylated truncated TRAF2 (residues 264-501) in 25 PBS.

- B. Preparation of assay plates:
- Coat with 120 µl of stock N-Avidin per well overnight at 4°C.
- Wash 2 times with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2 times with 200 µl PBS.
- 30 C. Assay:
- Add 40 µl assay buffer/well.
- Add 10 µl compound or extract.

- Add 10  $\mu$ l  $^{33}\text{P}$ -human c-IAP1 (20,000-25,000 cpm/0.1-10 pmoles/well =  $10^9$ - $10^{17}$  M final concentration).

- Shake at 25°C for 15 minutes.
- Incubate additional 45 minutes at 25°C.

5 - Add 40  $\mu$ l biotinylated truncated TRAF2 (0.1-10 pmoles/40  $\mu$ l in assay buffer)

- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200  $\mu$ l PBS.
- Add 150  $\mu$ l scintillation cocktail.

10 - Count in Topcount.

D. Controls for all assays (located on each plate):

- a. Non-specific binding
- b. Soluble (non-biotinylated truncated TRAF2) at 80% inhibition.

15 15 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those

20 20 of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

	SEQUENCE ID NO: 1, 2	h (human) c-IAP1	cDNA, protein
25	SEQUENCE ID NO: 3, 4	h c-IAP2	cDNA, protein
	SEQUENCE ID NO: 5, 6	h c-IAP1,2 repeat 1	protein, protein
	SEQUENCE ID NO: 7, 8	h c-IAP1,2 repeat 2	protein, protein
	SEQUENCE ID NO: 9, 10	h c-IAP1,2 repeat 3	protein, protein
	SEQUENCE ID NO: 11, 12	h c-IAP1,2 RING finger	protein, protein
30	SEQUENCE ID NO: 13, 14	m (murine) c-IAP	cDNA, protein